STIC-ILL

4/1501.A77
NPL

From: STIC-Biotech/ChemLib
Sent: Thursday, September 06, 2001 2:20 PM
To: STIC-ILL
Subject: FW: 09/276,935 reference request

-----Original Message-----
From: Pak, Michael
Sent: Thursday, September 06, 2001 2:19 PM
To: STIC-Biotech/ChemLib
Subject: 09/276,935 reference request

Dear STIC people:

Michael D. Pak
Art Unit 1646
CM1/10E13
305-7038
09/276,935
Needed ASAP.

Please send me copies of these references.
Please also copy the table of contents page of the journal issue for each reference.

1. Kliewer et al. (1998) Cell 92: 73-82.
2. Zhang et al. (1999) Arch. Biochem. Biophys. 368: 14-22.
3. Li et al. (1997) Endocrinology 138: 2347-2353.

Archives of Biochemistry and Biophysics
Vol. 368, No. 1, August 1, pp. 14–22, 1999
Article ID abbi.1999.1307, available online at http://www.idealibrary.com on IDEAL®

Rat Pregnane X Receptor: Molecular Cloning, Tissue Distribution, and Xenobiotic Regulation[1]

He Zhang, Edward LeCulyse,[2] Lan Liu, Maowen Hu, Lynn Matoney, Weizhu Zhu, and Bingfang Yan[3]
Department of Biomedical Sciences, University of Rhode Island, Kingston, Rhode Island 02881

Received April 9, 1999, and in revised form May 12, 1999

An orphan nuclear receptor, termed the pregnane X receptor (PXR), has recently been cloned from mouse and human and defines a novel steroid signaling pathway (*Cell* 92, 73–82, 1998; *Proc. Natl. Acad. Sci. USA* 95, 12208–122313, 1998). Transient cotransfection experiments demonstrate that the PXR responds to structurally dissimilar compounds and confers the induction of cytochrome P4503A (CYP3A), a subfamily of enzymes that involve the metabolism of two-thirds of drugs and other xenobiotics. In this report, we describe the molecular cloning, tissue distribution, and xenobiotic regulation of a rat PXR designated rPXR-1. rPXR-1 exhibits a 95% sequence identity with the mouse PXR, but only 79% identity with the human PXR, providing the molecular basis that rats and mice have a similar CYP3A induction profile but differ from humans. rPXR-1 gene was expressed abundantly in liver, intestine, and, to a lesser extent, kidney, lung, and stomach. The tissue distribution and the relative abundance of rPXR-1 mRNA among these tissues resemble those of CYP3A, suggesting that PXR is important not only for induction but also for constitutive expression of these enzymes. Xenobiotics known to induce liver microsomal enzymes showed differential effects on the rPXR-1 expression as determined by Northern blot analysis. Dexamethasone, for example, increased the accumulation of rPXR-1 mRNA, whereas troleandomycin slightly suppressed it. Compounds that increase PXR expression (inducers) and compounds that interact with PXR (ligands) likely have synergistic effects on CYP3A induction, which provides a novel molecular explanation for drug-drug interactions. © 1999 Academic Press

Key Words: **pregnane X receptor; pregnane A receptor; orphan nuclear receptor; cytochrome P4503A induction.**

Cytochrome P450 (CYP)[4] enzymes are a family of heme-containing proteins and rank first among the phase I biotransformation enzymes in terms of catalytic versatility and the number of xenobiotics they metabolize (1–3). Therefore, P450 enzymes determine the intensity and duration of action of drugs and play key roles in the detoxication and bioactivation of xenobiotics. P450 enzymes are present in all mammalian tissues with the highest concentration in liver endoplasmic reticulums (3). In each mammalian species, four families of P450 enzymes with a total of ~20 members are involved in xenobiotic metabolism (4). Among them, cytochrome P4503A (CYP3A) enzymes are the most abundant P450 enzymes and involve the metabolism of two thirds of xenobiotics (4).

Environmental factors are known to affect CYP3A activity by directly interacting with or regulating the expression of these enzymes (5–7). CYP3A enzymes have a broad substrate specificity; thus, most drugs may act as competitive inhibitors (2, 3). However, some drugs are shown to inactivate these enzymes by binding to the catalytically important moiety irreversibly (8, 9). For example, CYP3A4 converts macrolide anti-

[1] This work was partially supported by a New Investigator Award from the American Association of Colleges of Pharmacy and a grant from Rhode Island Foundation.

[2] Present address: Department of Drug Delivery and Disposition, School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599.

[3] To whom correspondence should be addressed. Fax: (401) 874-5048.

[4] Abbreviation used: AIDS, acquired immunodeficiency syndrome; CYP, cytochrome P450; DBD, DNA binding domain; HIV, human immunodeficiency virus; LBD, ligand binding domain; PXR, pregnane X receptor; SDS–PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TAD, N-terminal transactivating domain.

0003-9861/99 $30.00

biotics to metabolites that bind tightly to the heme moiety and permanently inactivate this enzyme, which accounts for the toxicity with terfenadine. Terfenadine is normally converted to an antihistaminic acid metabolite by two steps of CYP3A4 oxidations. But coadministration of macrolide antibiotics significantly elevates the plasma levels of terfenadine and leads to arrhythmias as a result of blocking cardiac potassium channels (10). Induction of CYP3A, like inhibition, may have profound clinical consequences. Chronic dosing with rifampicin, for example, markedly elevates CYP3A levels, which often results in a lower than expected plasma concentration of a drug, when this drug is a CYP3A substrate and is subsequently administered (5, 6). Accordingly, the administration of a usual drug dosage regimen may be therapeutically ineffective.

The induction of CYP3A enzymes is largely due to transcriptional activation (11, 12–14). Analyses of CYP3A promoters by DNase I footprinting locate a region that contains two copies of the AG(G/T)TCA motif. Recently, an orphan nuclear receptor designated the pregnane X receptor (PXR) has been cloned from mouse and human and shown to bind to this motif and confer CYP3A induction in response to the treatment with several xenobiotics (15–17). Structurally, PXR is similar to nuclear receptors, including a N-terminal transactivating domain (TAD), followed by a DNA binding domain (DBD), and a *C*-terminal ligand binding domain (LBD). The human and mouse PXRs have a 96% sequence identity in the DBD regions. However, the LBD regions are much less identical (~75%). Northern analyses show that PXR is primarily expressed in liver and intestine, two tissues that are known to contain high levels of CYP3A enzymes (15, 16).

In this report, we describe the molecular cloning, tissue distribution, and xenobiotic regulation of a rat PXR designated rPXR-1. rPXR-1 exhibits a 95% sequence identity with the mouse PXR, but only 79% identity with the human PXR, providing the molecular explanation that rats and mice have a similar CYP3A induction profile but differ from humans. rPXR-1 gene was expressed abundantly in liver, intestine, and, to a lesser extent, kidney, lung, and stomach. The tissue distribution and the relative abundance of rPXR-1 mRNA among these tissues resemble those of CYP3A, suggesting that PXR is important not only for induction and but also constitutive expression of these enzymes. Several xenobiotics known to induce liver microsomal enzymes showed differential effects on the rPXR-1 mRNA levels. Compounds such as perfluorodecanoic acid and isoniazid markedly increased the rPXR-1 mRNA levels. In contrast, compound such as troleandomycin slightly decreased the levels of rPXR mRNA. PXR inducers and ligands likely have synergistic effects on CYP3A induction, which provides a novel molecular explanation for drug–drug interaction.

MATERIALS AND METHODS

Chemicals and supplies. The rat liver cDNA library and the cDNA positive selection kit were purchased from GIBCO-BRL (Gaithersburg, MD). TRI REAGENT RNA extraction solution was from Sigma Chemical Co. (St. Louis, MO). Kits for *in vitro* transcription/translation or primer extension labeling were from Promega Corporation (Madison, WI). The isothermal DNA sequencing kit was purchased from Epicentre Technology Inc. (Cleveland, OH). [^{35}S]Methionine was purchased from NEN Life Science Products (Boston, MA). Sprague–Dawley rats (8–10 weeks old) were purchased from Charles River (Wilmington, MA). Chemicals used to treat rats were described elsewhere (18). Unless otherwise indicated, all other reagents were purchased from Fisher Scientific (Pittsburgh, PA).

Molecular cloning of rPXR-1. Molecular cloning was conducted with a cDNA-trapping procedure as described previously (19). Briefly, double-stranded phagemid DNA was isolated from a rat liver cDNA library and converted to ssDNA by a sequential digestion with Gene II and Exo III. The ssDNA was denatured at 95°C for 1 min and chilled in ice for 1 min. An oligonucleotide (ACAGTGGCTGTGAGCTTCCAG) based on the sequence of mouse PXR1 was synthesized by Genemed Synthesis, Inc. (South San Francisco, CA). The oligonucleotide (3 μg) was then biotinylated in a total volume of 25 μl with biotin-14-dCTP and terminal deoxynucleotidyl transferase. Hybridization between the ssDNAs (2.5 μg) from the liver library and the biotinylated oligonucleotide (20 ng) was conducted at 37°C for 1 h with the hybridization buffer provided with the kit. The hybridized ssDNAs were then captured by streptavidin-coated beads and repaired by a thermostable polymerase. The repaired ssDNAs were then transformed into HB101 bacteria. For sequencing, the phagemid DNA was isolated with a Spin Mini-prep kit (Qiagen, Inc., Chatsworth, CA) and sequenced with an Isothermal Sequencing kit. The initial sequences of the 3′ and 5′ ends were determined by vector primers as described previously (20). The complete sequence was determined by Genemed Synthesis, Inc. (South San Francisco, CA) based on the use of vector/internal synthetic primes on both strands.

In vitro transcription/translation. Rat PXR was synthesized with the TNT rabbit reticulocyte lysate coupled *in vitro* transcription/translation system (Promega, Madison, WI), essentially as described by the manufacturer. Rat PXR-1 plasmid (0.5 μg) was added to 24 μl reaction mixture containing 12.5 μl reticulocyte lysate, methionine-minus amino acid mixture (40 μM each), 20 units of RNasin, and 1 μl of [^{35}S]methionine (1,200 Ci/mmol at 10 mCi/ml). The reaction mixture was incubated at 30°C for 90 min and chased with cold methionine at a final concentration of 40 μM for 30 min. The reaction was terminated by adding an equal amount of 2× SDS–PAGE sample buffer (20% glycerol, 2% SDS, 0.025% bromophenol blue, 62.5 mM Tris–HCl at pH 6.8, 5% 2-mercaptoethanol). The *in vitro* translated products were denatured at 95°C for 3 min and subjected to SDS–PAGE. The synthesized rat PXR-1 was detected by autoradiography.

Animal treatment. To examine the effects of microsomal enzyme inducers on the expression of rat PXR, Sprague–Dawley rats (5 per group) were treated as described previously (18). Mature female rats received once daily injections of troleandomycin (500 mg/kg) or saline (5 ml/kg) for 4 consecutive days. Mature male rats were injected ip once daily with phenobarbital (80 mg/kg), isoniazid (200 mg/kg), clofibric acid (200 mg/kg), β-naphthoflavone (100 mg/ml), 3-methylcholanthrene (27 mg/kg), pregnenolone-16α-carbonitrile (50 mg/kg), or dexamethasone (50 mg/kg). The first three inducers were dissolved in saline, whereas the others were dissolved or suspended in corn oil. Perfluorodecanoic acid (40 mg/kg) was dissolved in corn oil

and administered as a single ip injection to male rats. All rats were housed in an AAALAC-accredited facility and allowed free access to Purina Rodent Chow 5001 and water.

Northern blotting. Total RNA from control or xenobiotic-treated Sprague–Dawley adult rats was isolated with a TRI REAGENT RNA extraction solution according to the instruction by the manufacturer. RNA samples from each group of rats were pooled and the concentration was determined from the absorbance at 260 nm, with 1 O.D. unit equal to 40 μg/ml. Total RNA (20 μg) was fractionated by electrophoresis in 1.2% agarose gels containing 2.2 M formaldehyde. Fractionated RNA was blotted to a Nytran nylon membrane (Schleicher & Schuell, Keene, NH) with a vacuum-blotting system (Pharmacia, Piscataway, NJ) as described previously (20). The membrane was incubated overnight in 5 ml of hybridization buffer (250 mM sodium phosphate buffer, pH 7.2, 1 mM EDTA, 7% SDS, and 0.01% bovine serum albumin) containing a ^{32}P-labeled cDNA probe (~2 × 10^5 cpm). The hybridization was conducted at 68°C in a rotating oven. The cDNA probe was prepared by radiolabeling the rat PXR (only the insert) with a Prime-a-Gene system as described previously (20). After hybridization, the membrane was washed at 68°C for 1 h in hybridization buffer (2 × 30 min) and for 1 h in 40 mM sodium phosphate buffer, pH 7.2, containing 1 mM EDTA and 0.5% SDS (2 × 30 min). A Kodak X-OMAT X-ray film was exposed to the membrane at −70°C with the aid of a DuPont Cronex Lighting intensifying screen until an appropriately exposed autoradiogram was obtained (~4 days). To normalize the abundance of 28S rRNA contained in each sample, the same membrane was stripped by boiling 2 × 15 min and reprobed with an oligonucleotide (hybridize with 28S rRNA) ^{32}P-radiolabeled with T4 polynucleotide kinase as described previously (20).

RESULTS

Molecular Cloning of rPXR-1

Based on a recent report on the cloning of mouse PXR (15), an oligonucleotide was synthesized and biotinylated. The biotinylated oligonucleotide was then used to screen a rat liver cDNA library with a GeneTrapp cDNA Positive Selection System. Screening of this library yielded ~100 individual clones, and 16 of them were sequenced at the 5′ and 3′ ends. Sequencing analysis revealed that four of these clones contained inserts that had a high degree of sequence identity with the mouse PXR1. The largest clone, designated rPXR-1, was completely sequenced. As shown in Fig. 1, rPXR-1 spanned 1734 bp and contained an open reading frame encoding 431 amino acids, followed by a termination codon (TGA). rPXR-1 had 217 and 220 nucleotides of untranslated sequences at both the 5′ and 3′ ends, respectively. The 3′ untranslated region contained a polyadenylation signal (AATAAA), and a poly(A) tail began 15 bp after this signal.

Like its counterpart in mouse and human (15–17), rat PXR has three functional domains: TAD, DBD, and LBD. Overall, rat and mouse PXRs exhibit a higher sequence identity (~90%) than with the human PXR (~80%) at both nucleotide and amino acid levels (Fig. 2). The DBD (amino acid 38–104) is highly conserved and has a >95% sequence identity among three PXRs, suggesting that they interact with the same or a similar DNA response element. In contrast, the LBD (amino acid 138–431) is relatively diverse and has a ~78% sequence identity, suggesting that they have a different ligand specificity (Fig. 2).

In Vitro Transcription/Translation

The rat liver cDNA library was constructed with the phagemide SPORT vector, which had a cytomegalovirus (CMV) promoter for eukaryotic expression and SP6/T7 promoters for prokaryotic expression. In order to determine the translated products of rPXR-1, an *in vitro* translation experiment was conducted with a TNT rabbit reticulocyte lysate coupled *in vitro* transcription/translation system. As shown in Fig. 3, rPXR-1 yielded three major products with a molecular weight of 58-, 51-, and 42-kDa, respectively. The 51-kDa protein was the dominant product, but significant amounts of the 58- or 42-kDa protein were produced. Use of a smaller amount of rPXR-1 plasmid or longer incubation for reaction chase with cold methionine caused little change in the ratio of the abundance among these three products (results not shown). The 58-kDa protein was likely resulted from posttranslational modification or translationally initiated from non-AUG codons upstream of the AUG codon indicated in Fig. 1. The 42-kDa protein, on the other hand, represented translation initiation at methionine-69, a codon surrounded by a favorable Kozak sequence (22). The 42-kDa protein had an intact LBD but a truncated DBD; thus, this protein likely binds to the ligands but confers little transactivation activity.

Tissue Distribution of rPXR-1

The tissue expression pattern of rPXR-1 was determined by Northern blot analysis. Total RNA was isolated from various tissues of adult male rats. rPXR-1 was expressed abundantly in liver, intestine, and, to a lesser extent, kidney, lung, and stomach (Fig. 4). This distribution of rPXR-1 differs from that of hPXR but resembles that of mPXR (15–17). In contrast to mPXR, rPXR-1 mRNA was relatively abundant in kidney, lung, and stomach based on its abundance in liver and intestine. Such differences likely resulted from the sample preparation. Poly(A) RNA was used to determine the tissue distribution for mPXR, whereas total RNA was used for rPXR-1. Selection of poly(A) RNA was based on polyadenylation. Differences in polyadenylation processing on PXR mRNA among these tissues probably caused a disproportional enrichment during poly(A) RNA preparation. In addition, rPXR-1 mRNA resembles CYP3A enzymes in both tissue distribution and relative abundance among these tissues, suggesting that PXR is important not only for induc-

```
1     GAGGACATTGGCCTCTCCCTGGGAGCCTGACCTCGGCCCATACGAAACACCAAAGACTTAAGCTGAGACTT 71
72    CTGTATGCAACCAGGAGGCTGCAGCAGGGGTGCTGAGCTCTGGGCAGAAACATCATCCCTCACCCTTCAAA 142
143   GTGGACCCCAAGGGAGAAATCCAACAAAAGCAGTGGCCACCTAACAGTCCAGGACACACAGATGTAAACCT 213
214   GGAG    ATG AGA CCT GAG GAG AGG TGG AAC CAT GTT GGC CTT GTA CAA CGT GAA 265
1               Met Arg Pro Glu Glu Arg Trp Asn His Val Gly Leu Val Gln Arg Glu 16
266   GAA GCA GAT TCT GTC TTG GAA GAG CCT ATC AAC GTA GAT GAG GAA GAT GGA GGT 319
17    Glu Ala Asp Ser Val Leu Glu Glu Pro Ile Asn Val Asp Glu Glu Asp Gly Gly 34
320   CTT CAA ATC TGC CGT GTA TGT GGG GAC AAG GCC AAT GGC TAT CAC TTC AAT GTC 373
35    Leu Gln Ile Cys Arg Val Cys Gly Asp Lys Ala Asn Gly Tyr His Phe Asn Val 52
374   ATG ACC TGT GAA GGA TGT AAG GGA TTT TTC AGA AGG GCC ATG AAA CGC AAT GTC 427
53    Met Thr Cys Glu Gly Cys Lys Gly Phe Phe Arg Arg Ala Met Lys Arg Asn Val 70
428   CGG CTG AGG TGC CCC TTC CGC AAG GGG ACC TGC GAG ATC ACC CGG AAG ACC CGA 481
71    Arg Leu Arg Cys Pro Phe Arg Lys Gly Thr Cys Glu Ile Thr Arg Lys Thr Arg 88
482   CGG CAG TGC CAG GCC TGC CGT TTG CGC AAG TGC CTG GAG AGT GGC ATG AAG AAA 535
89    Arg Gln Cys Gln Ala Cys Arg Leu Arg Lys Cys Leu Glu Ser Gly Met Lys Lys 106
536   GAG ATG ATC ATG TCT GAT GCC GCT GTG GAA CAG AGG CGG GCC TTG ATC AAG AGG 589
107   Glu Met Ile Met Ser Asp Ala Ala Val Glu Gln Arg Arg Ala Leu Ile Lys Arg 124
590   AAG AAG AGG GAA AAG ATT GAG GCT CCA CCG CCT GGA GGG CAG GGG CTG ACA GAA 643
125   Lys Lys Arg Glu Lys Ile Glu Ala Pro Pro Pro Gly Gly Gln Gly Leu Thr Glu 142
644   GAA CAG CAG GCG CTG ATC CAG GAG CTG ATG GAC GCT CAG ATG CAA ACC TTT GAC 697
143   Glu Gln Gln Ala Leu Ile Gln Glu Leu Met Asp Ala Gln Met Gln Thr Phe Asp 160
698   ACA ACT TTC TCC CAC TTC AAG GAT TTC CGG CTA CCT GCG GTG TTT CAC AGT GAC 751
161   Thr Thr Phe Ser His Phe Lys Asp Phe Arg Leu Pro Ala Val Phe His Ser Asp 178
752   TGC GAG CTT CCG GAG GTT CTG CAG GCC TCA CTG TTG GAA GAC CCT GCC ACA TGG 805
179   Cys Glu Leu Pro Glu Val Leu Gln Ala Ser Leu Leu Glu Asp Pro Ala Thr Trp 196
806   AGT CAA ATC ATG AAA GAC AGT GTT CCA ATG AAG ATC TCT GTG CAG CTG CGC GGA 859
197   Ser Gln Ile Met Lys Asp Ser Val Pro Met Lys Ile Ser Val Gln Leu Arg Gly 214
860   GAA GAC GGC AGC ATC TGG AAC TAC CAA CCT CCC TCC AAG AGC GAC GGG AAA GAG 913
215   Glu Asp Gly Ser Ile Trp Asn Tyr Gln Pro Pro Ser Lys Ser Asp Gly Lys Glu 232
914   ATC ATC CCC CTC CTG CCG CAC CTG GCC GAT GTG TCA ACC TAC ATG TTC AAG GGC 967
233   Ile Ile Pro Leu Leu Pro His Leu Ala Asp Val Ser Thr Tyr Met Phe Lys Gly 250
968   GTC ATC AAC TTC GCC AAA GTC ATA TCC CAC TTC AGG GAG CTG CCT ATC GAG GAC 1021
251   Val Ile Asn Phe Ala Lys Val Ile Ser His Phe Arg Glu Leu Pro Ile Glu Asp 268
1022  CAG ATC TCC CTG CTG AAG GGG GCC ACT TTC GAG ATG TGC ATC CTG AGG TTC AAC 1075
269   Gln Ile Ser Leu Leu Lys Gly Ala Thr Phe Glu Met Cys Ile Leu Arg Phe Asn 286
1076  ACG ATG TTC GAC ACG GAA ACA GGA ACC TGG GAG TGC GGT CGG CTG GCT TAC TGC 1129
287   Thr Met Phe Asp Thr Glu Thr Gly Thr Trp Glu Cys Gly Arg Leu Ala Tyr Cys 304
1130  TTC GAA GAC CCT AAT GGC GGC TTC CAG AAG CTC CTG CTG GAC CCG TTG ATG AAA 1183
305   Phe Glu Asp Pro Asn Gly Gly Phe Gln Lys Leu Leu Leu Asp Pro Leu Met Lys 322
1184  TTC CAC TGC ATG CTG AAG AAG CTA CAG CTG CGT GAG GAG GAG TAC GTG CTG ATG 1237
323   Phe His Cys Met Leu Lys Lys Leu Gln Leu Arg Glu Glu Glu Tyr Val Leu Met 340
1238  CAG GCC ATC TCC CTC TTC TCC CCA GAT CGC CCT GGC GTG GTT CAA CGT AGC GTG 1291
341   Gln Ala Ile Ser Leu Phe Ser Pro Asp Arg Pro Gly Val Val Gln Arg Ser Val 358
1292  GTA GAC CAG CTG CAG GAG CGA TTT GCC CTC ACC CTG AAG GCC TAC ATC GAG TGT 1345
359   Val Asp Gln Leu Gln Glu Arg Phe Ala Leu Thr Leu Lys Ala Tyr Ile Glu Cys 376
1346  AGT CGG CCC TAT CCT GCA CAC AGG TTC CTG TTC CTG AAG ATC ATG GCT GTC CTC 1399
377   Ser Arg Pro Tyr Pro Ala His Arg Phe Leu Phe Leu Lys Ile Met Ala Val Leu 394
1400  ACC GAG CTG CGC AGT ATC AAT GCC CAG CAG ACC CAG CAG CTA CTG CGC ATC CAG 1453
395   Thr Glu Leu Arg Ser Ile Asn Ala Gln Gln Thr Gln Gln Leu Leu Arg Ile Gln 412
1454  GAC ACG CAC CCC TTT GCC ACA CCT CTC ATG CAG GAG TTA TTC AGC AGC ACG GAC 1507
413   Asp Thr His Pro Phe Ala Thr Pro Leu Met Gln Glu Leu Phe Ser Ser Thr Asp 430
1508  GGC TGA       GTGGCTGCCCCTGAGTGGAGATCTCACGGAGCAGCCAGACCCAGATGTTCTGAATTGC 1571
431   Gly ***                                                               431
1572  CACTTCTAGGGCTATCAGATGGACACACTGATAACTAACAATGCCTCTGTCTGCAGCTGGCTAGCATTTCT 1642
1643  CAGGAAAAGGACACAGGAGCTCAGCCTGTGGAAAGTGCTGGCCTAGAAATTAGACCATCTCTGTGGTTGGG 1713
1714  AATAAACCTTCAAATCCTGCTAAAAAAAAAAAAAAAAAAAAAA                           1755
```

FIG. 1. Nucleotide sequences and predicted amino acid sequences of rPXR-1. Both nucleotide and predicted amino acid sequences are countered on both sides. The amino acid is numbered beginning with the first UAG codon, which yields the 51-kDa protein (Fig. 2). The polyadenylation signal is underlined. The Accession No. is AF106005.

```
rPXR-1 MRPEERWNHV GLVQREEADS VLEEPINVDE EDGGLQICRV CGDKANGYHF NVMTCEGCKG  60
mPXR-1 *****S*SR* ****C***** A*******E* ********** ********** **********  60
hPXR   V**K*S***A DFVHC*DTE* *PGK*SVN** *V**P***** *****T**** **********  63

rPXR-1 FFRRAMKRNV RLRCPFRKGT CEITRKTRRQ CQACRLRKCL ESGMKKEMIM SDAAVEQRRA 120
mPXR-1 ********** ********** ********** ********** ********** ********** 120
hPXR   *********A *********A ********** ********** ********** **E***E*** 123

rPXR-1 LIKRKKREKI EAPPPGGQGL TEEQQALIQE LMDAQMQTFD TTFSHFKDFR LPAVFHSDCE 180
mPXR-1 ********** ********** ********** ********** ********** *******G** 180
hPXR   ******S*RT GTQ*L*V*** ****RMM*R* ******K*** *******N** **G*LS*G** 183

rPXR-1 LPEVLQASLL EDPATWSQIM KDSVPMKISV QLRGEDGSIW NYQPPSKSDG KEIIPLLPHL 240
mPXR-1 ***F****** ********** **R******L ********** ********** ********** 240
hPXR   ***S***PSR *EA*K***VR **LCSLKV*L ********V* **K**AD*G* ***PS****M 243

rPXR-1 ADVSTYMFKG VINFAKVISH FRELPIEDQI SLLKGATFEM CILRFNTMFD TETGTWECGR 300
mPXR-1 ********** *********Y **D******* ********** ********** ********** 300
hPXR   **M******* I*S******Y **D******* ******A**L *Q*****V*N A********** 303

rPXR-1 LAYCFEDPNG GFQKLLLDPL MKFHCMLKKL QLREEEYVLM QAISLFSPDR PGVVQRSVVD 360
mPXR-1 ********** ********** ********** **HK****** ********** ********** 360
hPXR   *S**L**TA* ***Q***E*M L***Y***** **H******* ********** ***L*HR*** 363

rPXR-1 QLQERFALTL KAYIECSRPY PAHRFLFLKI MAVLTELRSI NAQQTQQLLR IQDTHPFATP 420
mPXR-1 ********** ********** ********** ********** ********** ***S****** 420
hPXR   ****Q**I** *S****N**Q ********** **M******* ***H**R*** ***I****** 423

rPXR-1 LMQELFSSTD G                                                       431
mPXR-1 ********** *                                                       431
hPXR   ******GI*G S                                                       434
```

FIG. 2. Comparison of the deduced amino acid sequences of rPXR-1, mPXR1, and hPXR. Amino acids are shown in single letters. The top, middle, and bottom lines show the deduced amino acid sequences of rPXR-1, mPXR (15), and hPXR (17), respectively. Deletions of Leu_1, Glu_2, and Ala_{12} in hPXR are introduced for maximum alignment. hPXR is identical to hPAR-1 (16).

tion and but also for constitutive expression of these enzymes (7, 24, 25).

Effects of Xenobiotic Treatment on the Levels of mRNA Encoding rPXR-1

Several xenobiotics known to induce liver CYP450 enzymes were examined for their ability to regulate the levels of mRNA encoding rPXR-1. These results are shown in Fig. 5. The CYP1A inducers, β-naphthoflavone and 3-methylcholanthrene, slightly increased the rPXR mRNA levels. Similarly the classic CYP2B inducer, phenobarbital, caused a slight increase in the rPXR mRNA levels although this inducer is known to moderately induce CYP3A (3). The CYP2E inducer, isoniazid, significantly increased the rPXR mRNA levels. The CYP3A inducers, dexamethasone, pregnenolone-16α-carbonitrile, and troleandomycin, had differential effects. The former two compounds moderately increased the accumulation of rPXR-1 mRNA (1- to 3-fold), whereas the third compound slightly decreased it. The CYP4A inducers, clofibrate and perfluorodecanoic acid, which are known as peroxisome proliferators, caused a 2- and 10-fold increase in the accumulation of rPXR-1 mRNA, respectively. Some peroxisome proliferators are found to moderately induce CYP3A enzymes (26). The induction of rPXR-1 by isoniazid is clinically significant. HIV-positive patients usually receive antiviral therapy (e.g., protease inhibitors) and at the same time receive isoniazid and rifampicin to prevent pulmonary tuberculosis (27). Rifampicin is a potent CYP3A inducer in humans and most protease inhibitors are metabolized by CYP3A enzymes (27). Therefore, coadministration of isoniazid and rifampicin may cause synergistic induction on CYP3A enzymes, which rapidly eliminate protease inhibitors and lower the effectiveness of antiviral therapy.

DISCUSSION

CYP3A enzymes involve the metabolism of more than two-thirds of drugs and other xenobiotics. Induction of CYP3A enzymes is known as an important

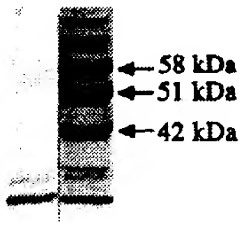

FIG. 3. *In vitro* transcription/translation of rPXR-1. Rat PXR plasmid (0.5 μg) was added to 24 μl reaction mixture containing 12.5 μl reticulocyte lysate, methionine-minus amino acid mixture (40 μM each), 20 units of RNasin, and 1 μl of [^{35}S]methionine (1200 Ci/mmol at 10 mCi/ml). The reaction mixture was incubated at 30°C for 90 min and terminated by adding an equal amount of 2× SDS–PAGE sample buffer. The *in vitro* translated products were denatured at 95°C for 3 min and subjected to SDS–PAGE. The synthesized rat PXR was detected by autoradiography.

contributing factor to failed drug therapy or severe toxicity as a result of drug–drug interaction or bioactivation. The induction of CYP3A enzymes is largely due to transcriptional activation (9, 10). Analyses of CYP3A promoters locate a region upstream to the transcriptional initiation site that contains two copies of the AG(G/T)TCA motif, a characteristic binding motif of many nuclear receptors that include the receptors for thyroid hormone, vitamin D3, retinoic acid, and 9-*cis*-retinoic acid, and the orphan receptors (31, 32). Recently, an orphan nuclear receptor designated PXR has been cloned from mouse and human and shown to bind to this motif and confer CYP3A induction in response to the treatment with several xenobiotics (15–17). In this report, we describe the molecular cloning, tissue distribution, and xenobiotic regulation of the rat counterpart: rPXR-1.

One of the features regarding CYP3A induction is the marked species differences. Rifampicin, for example, causes CYP3A induction by as much as 10-fold in humans (33). In contrast, this antibiotic causes only moderate induction (~2-fold) in mice when a dose of 40 mg/kg is given once daily for 3 days (34). Rats are far less sensitive to the induction by rifampicin. No induction was observed when a dose of 200 mg/kg was given once daily for 7 days (35). Transient cotransfection experiments demonstrate that the mouse PXR confers 2-fold induction and the human PXR confers 7-fold induction in response to rifampicin, suggesting that PXR is largely responsible for the species differences (15–17). The DBD is highly conserved among the PXRs; thus, the species difference is likely due to the differences in the LBD. Alignment analysis reveals that rPXR has only nine amino acid substitutions compared with the mouse PXR in the LBD (Fig. 6), and they are likely responsible for the lower responsiveness of rats to rifampicin. Compared with the human PXR, five of the nine amino acids in mouse PXR are conserved in human PXR (in bold), and the remaining four residues, Phe_{184}, Arg_{203}, Lys_{334}, and Ser_{414}, are substi-

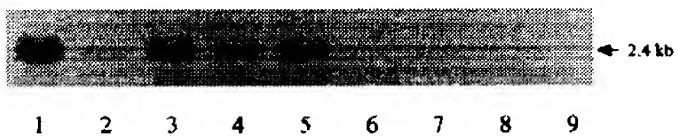

FIG. 4. Tissue distribution of mRNA encoding rPXR-1. The tissue distribution of mRNA encoding rPXR-1 was determined by Northern blotting, as described under Materials and Methods. Total RNA was isolated from liver, kidney, intestine, testis, lung, heart, brain, spleen, and stomach from 12-week-old male Sprague–Dawley rats. Tissue RNA from five rats were pooled, and an aliquot (20 μg) was subjected to 2.2 M formaldehyde-agarose gel electrophoresis and transferred to a Nytran nylon membrane with a vacuum-blotting system. The mRNA encoding rPXR-1 was detected with ^{32}P-labeled cDNA probe.

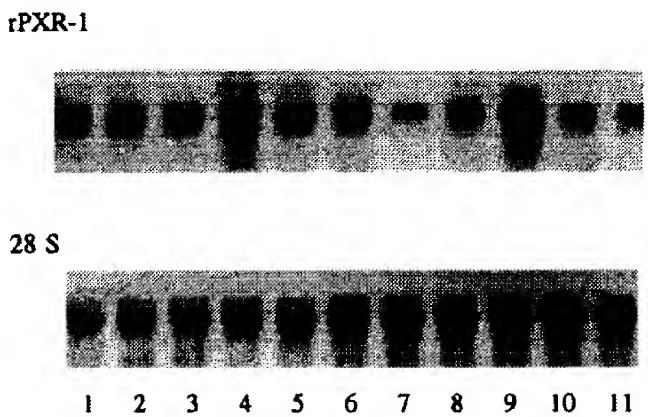

FIG. 5. Effects of treating Sprague–Dawley rats with various xenobiotics on the levels of mRNA encoding rPXR-1. Male rats (10-weeks old, five per group) and female rats (troleandomycin only) were treated with various xenobiotics at dosages known to induce liver microsomal cytochrome P450, as described under Materials and Methods. Pooled total RNA (20 μg) was subjected to 2.2 M formaldehyde–agarose gel electrophoresis and transferred to a Nytran nylon membrane with a vacuum-blotting system. The mRNA encoding rPXR-1 was detected with ^{32}P-labeled cDNA probe. To normalize the abundance of 28S rRNA contained in each sample, the membrane was stripped by boiling 2 × 15 min and reprobed with a ^{32}P-labeled oligonucleotide as described previously (20), which is shown at the bottom.

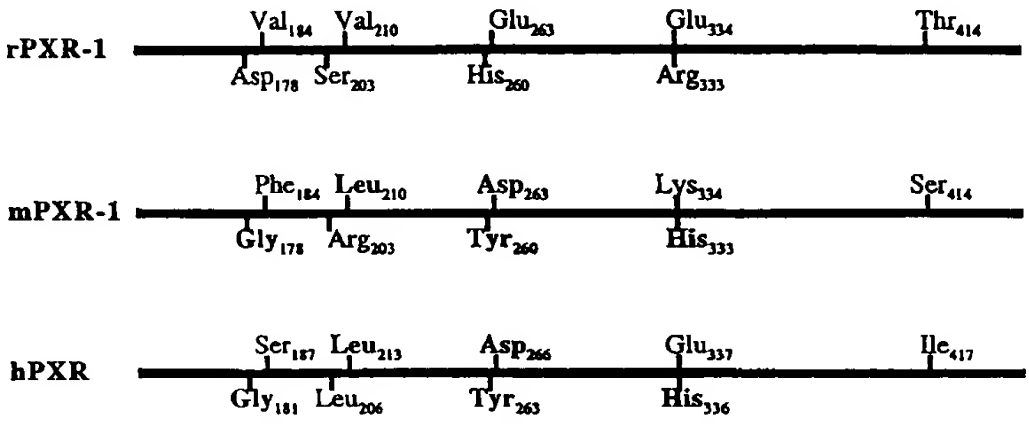

FIG. 6. Substitutions of amino acids in the ligand binding domain between rPXR-1 and mPXR-1. The amino acid sequences deduced for the ligand binding domain of rPXR-1 (top), mPXR-1 (middle), and hPXR (bottom) are represented by solid lines. The substitutions of amino acids between rPXR-1 and mPXR-1 are specified. The substitutions that are conserved in hPXR are in bold. The deduced amino acids of rPXR are based on this report, while those for mPXR and hPXR are based on the reports by Kliewer *et al.* (15) and Lehmann *et al.* (17), respectively.

tuted by Ser_{414}, Leu_{203}, Glu_{334}, and Ile_{414}, respectively (Fig. 5). The five amino acids conserved between mouse and human PXRs likely support the interaction between PXR and rifampicin, which, in turn, supports CYP3A induction. The remaining four substitutions (Phe/Ser_{184}, Arg/Leu_{203}, Lys/Glu_{334}, and Ser/Ile_{414}) result in drastic changes in either hydrophobicity, the net charge, or both. Thus, these substitutions likely contribute to the lower responsiveness of mice to rifampicin compared with that of humans (Fig. 6).

The second feature regarding CYP3A induction is the structural diversity of inducers (3). CYP3A inducers include a wide range of drugs such as anticonvulsant agents (e.g., phenytoin), antibiotics (e.g., rifampicin and erythromycin), imidazole antifungals (e.g., clotrimazole), barbiturates (e.g., phenobarbital), cholesterol-lowering agents (e.g., lovastatin), and steroids (e.g., dexamethasone). Both glucocorticoids and antaglucocorticoids are shown to induce CYP3A (6, 7). Some pesticides such as *trans*-nonachlor are also inducers of CYP3A enzymes (8). Some of these compounds have been assayed in transient transfection experiments with the mouse or human PXR and have been shown to increase the activity of the reporter enzyme (15–17), suggesting that these compounds are PXR ligands. In this report, we present evidence that some xenobiotics such as PFDA actually increase the rPXR-1 mRNA levels (Fig. 5), and presumably increase the levels of the rPXR-1 protein as well. PFDA belongs to a family of peroxisome proliferators, and some of these compounds are weak inducers of CYP3A enzymes (26). Whether PFDA induces CYP3A in the dose described here remains to be determined. Nevertheless, the increased expression makes the PXR available for endogenous or xenobiotic ligands and causes CYP3A induction. Therefore, xenobiotics may induce CYP3A enzymes by acting as PXR inducers, PXR ligands, or both. This classification provides a molecular explanation for the structural diversity of CYP3A inducers and a novel mechanism that contributes to drug–drug interactions.

The third feature regarding CYP3A induction is interindividual variation. Rifampicin, for example, causes an increase in CYP3A expression by as much as 10-fold in some cultures of primary hepatocytes. However, in other cultures, induction is not apparent and even suppression is observed (36). Hepatocyte cultures that respond well to some CYP3A inducers may not necessarily respond to other CYP3A inducers. For example, hepatocytes from two individuals have a similar responsiveness to pregnenolone-16α-carbonitrile, but exhibit a 3-fold difference in response to rifampicin (36–38). Several possibilities may contribute to these variations. First, a polymorphism in human PXR may exist. As shown in Fig. 6, a few amino acids likely determine the responsiveness to rifampicin-mediated induction, and one or more substitutions of these residues may have a profound effect on CYP3A induction. Second, multiple forms of PXR resulting from alternative splicing or the existence of multiple genes have a different ligand specificity, and the relative levels of each form determine the overall induction by an inducer. Variants have been identified in both humans and mice (15, 16). The mouse variant, compared with the mPXR-1, is a much weaker transactivator in response to a panel of CYP3A inducers. Finally, we have demonstrated with *in vitro* transcription/translation assays that rPXR-1 produces three distinct products (Fig. 3), and a similar observation has been made with hPXR-1. The relative amount of each translated product likely affects the overall induction of CYP3A in response to an inducer.

29. Miranda, C. L., Reed, R. L., Guengerich, F. P., and Buhler, D. R. (1991) *Carcinogenesis* **12,** 515–519.
30. Chung, W., and Buhler, D. R. (1994) *Toxicol. App. Pharmacol.* **127,** 314–319.
31. Pichard, L., Fabre, I., Fabre, C., Domergue, J., Aubert, B. S., Mourad, G., and Maurel, P. (1990) *Drug Metab. Dipos.* **18,** 595–606.
32. Heuman, D. M., Gallagher, E. J., Barwick, J. L., Elshourbagy, N. A., and Guzelian, P. S. (1982) *Mol. Pharmacol.* **21,** 753–760.
33. Benedetti, M. S., and Dostert, P. (1994) *Environ. Health Perspect.* **102**(Suppl), 101–105.
34. Tredger, J. M., Smith, H. M., Powell-Jackson, P. R., Davis, M., and Williams (1981) *Biochem. Pharmacol.* **30,** 1043–1051.
35. Adachi, Y., Nanno, T., Yamashita, M., Ueshima, S., and Yamamoto, T. (1985) *Gastroenterol. Jpn.* **20,** 104–110.
36. Kostrubsky, V. E., Lewis, L. D., Strom, S. C., Wood, S. G., Schuetz, E. G., Schuetz, J. D., Sinclair, P. R., Wrighton, S. A., and Sinclair, J. F. (1998) *Arch. Biochem. Biophys.* **355,** 131–136.
37. Kocarek, T. A., Shuetz, E. G., Strom, S. C., Fisher, R. A., and Guzelian, P. S. (1995) *Drug Metab. Dispos.* **23,** 415–421.
38. Lake, B. G., Ball, S. E., Renwick, A. B., Tredger, J. M., Kao, J., Beamand, J. A., and Price, R. J. (1997) *Xenobiotica* **27,** 1165–1173.
39. Jackson, D. A., Collier, C. D., Oshima, H., and Simons, S. S. (1998) *J. Steroid Biochem. Mol. Biol.* **66,** 79–91.
40. Reid, I. R. (1997) *Eur. J. Endocrinol.* **137,** 209–217.
41. Chedid, M., Hoyle, J. R., Csaky, K. G., and Ribin, J. S. (1996) *Endocrinology* **137,** 2232–2237.
42. Iyer, A. M., Brook, S. M., and Sapolsky, R. M. (1998) *Brain Res.* **808,** 305–309.
43. Collier, S. D., Wu, W., and Pruett, S. B. (1998) *Toxicol. Appl. Pharmacol.* **148,** 176–182.

In addition to pharmacological and toxicological significance, CYP3A induction likely has physiological relevance. Glucocorticoids, for example, are both substrates and inducers of CYP3A enzymes (3, 7). However, a 100-fold greater concentration of these compounds is required to elicit a maximum CYP3A induction than is required to activate the glucocorticoid receptor (primary physiological function) (15–17, 37, 39). Such high concentrations required for CYP3A induction are usually seen in stress situations. High levels of glucocorticoid hormones have been found to cause tissue injury (40–42). In mice, stress-induced glucocorticoids markedly increase apoptotic rate of immunocytes (~5-fold) in the spleen (43). Induction of CYP3A by stress levels of glucocorticoids likely expedites the elimination of these hormones and prevents further tissue injury. Therefore, the PXR-mediated CYP3A induction provides a metabolizing feedback loop for corticosteroids, and plays a key role in maintaining steroid hormone homeostasis.

In summary, we report the molecular cloning, tissue distribution, and xenobiotic regulation of a rat PXR. The rat PXR is highly similar to mouse PXR-1 and differs from human PXR, particularly in the ligand binding domain. The similarity of rat PXR with mouse PXR in conjunction with its difference from human PXR provides a molecular basis regarding the species differences in CYP3A induction. PXR and CYP3A enzymes share a similar tissue expression pattern, suggesting that PXR is important not only for the induction but also for the constitutive expression of CYP3A enzymes. The expression of PXR can be regulated by xenobiotics, therefore, drugs or other xenobiotics induce CYP3A enzymes by acting as PXR inducers, PXR ligands or both. PXR ligand and PXR inducers may have synergistic effects on CYP3A induction, providing a novel mechanism for drug–drug interactions. Such interactions are particularly relevant to the therapy of AIDS patients. HIV-positive patients usually receive antiviral therapy (e.g., protease inhibitors) and, at the same time, receive isoniazid and rifampicin to prevent pulmonary tuberculosis. Rifampicin is known as a potent CYP3A inducer (PXR ligand) in humans and most protease inhibitors are metabolized by CYP3A enzymes. We demonstrate in this report that isoniazid causes a drastic induction of rPXR-1. Therefore, coadministration of isoniazid and rifampicin may cause synergistic induction on CYP3A enzymes, which rapidly eliminate protease inhibitors and lower the effectiveness of antiviral therapy.

REFERENCES

1. Gonzalez, F. J. (1989) *Pharmacol. Rev.* **40**, 243–288.
2. Guengerich, F. P. (1991) *J. Biol. Chem.* **266**, 10091–10022.
3. Parkinson, A. (1995) *in* The Casarett & Doull's Toxicology, the Basic Science of Poisons (Klaassen, C. D., Eds.), pp. 139–162, McGraw–Hill, New York.
4. Lewis, D. F., Watson, E., and Lake, B. G. (1998) *Mutat. Res.* **410**, 245–270.
5. Thummel, K. E., and Wilkinson, G. R. (1998) *Annu. Rev. Pharmacol. Toxicol.* **38**, 389–430.
6. Wilkinson, G. R. (1996) *J. Pharmacokin. Biopharm.* **24**, 475–490.
7. Kamisky, L. S., and Fasco, M. (1992) *Toxicology* **21**, 407–422.
8. Franklin, M. R. (1995) *Drug Metab. Dispos.* **23**, 1379–1382.
9. Witkamp, R. F., Nijmeijer, S. M., Monshouwer, M., and van Miert, A. S. J. P. A. M. (1995) *Drug Metab. Dispos.* **23**, 542–547.
10. Kivsito, K. T., Neuvonen, P. J., and Klotz, U. (1994) *Clin. Pharmacokin.* **27**, 1–5.
11. Barwick, J. L., Quattrochi, L. C., Mills, A. S., Potenza, C., Tukey, R. H., and Guzelian, P. S. (1996) *Mol. Pharmacol.* **50**, 10–16.
12. Schuetz, E. G., Schuetz, J. D., Strom, S. C., Thompson, M. T., Fisher, R. A., Molowa, D. T., Li, D., and Guzelian, P. S. (1992) *Hepatology* **18**, 1254–1262.
13. Hostetler, K. A., Wrighton, S. A., Molowa, D. T., Thomas, P. E., Levin, W., and Guzelian, P. S. (1989) *Mol. Pharmacol.* **35**, 279–285.
14. Komori, M., and Oda, Y. (1994) *J. Biochem.* **116**, 114–120.
15. Kliewer, S. A., Moore, J. T., Wade, L., Staudinger, J. L., Watson, M. A., Jones, S. A., McKee, D. D., Oliver, B. B., Willson, T. M., Zetterström, R. H., Perlmann, T., and Lehmann, J. M. (1998) *Cell* **92**, 73–82.
16. Lehmann, J. M., McKee, D. D., Watson, M. A., Willson, T. M., Moore, J. T., and Kliewer, S. A. (1998) *J. Clin. Invest.* **102**, 1016–1032.
17. Bertilsson, G., Heidrich, J., Svensson, K., Åsman, M., Jendeberg, L., Sydow-Bäckman, M., Ohlsson, R., Postlind, H., Blomquist, P., and Berkenstam, A. (1998) *Proc. Natl. Acad. Sci. USA* **95**, 12208–12213.
18. Yan, B., Yang, D., and Parkinson, A. (1995) *Arch. Biochem. Biophys.* **317**, 222–234.
19. Hu, M., and Yan, B. (1999) *Anal. Biochem.* **266**, 233–235.
20. Yan, B., Yang, D., and Parkinson, A. (1996) *J. Biol. Chem.* **270**, 19128–19134.
21. Yan, B., Yang, D., Brady, M., and Parkinson, A. (1994) *J. Biol. Chem.* **269**, 29688–29696.
22. Kozak, M. (1991) *J. Biol. Chem.* **266**, 19867–19870.
23. Debri, K., Boobis, A., Davies, D. S., and Edwards, R. J. (1995) *Biochem. Pharmacol.* **50**, 2047–2056.
24. Simmons, D. L., and Kasper, C. B. (1989) *Arch. Biochem. Biophys.* **271**, 10–20.
25. Voigt, J. M., Kawabata, T. T., Burke, J. P., Martin, M. V., Guengerich, F. P., and Baron, J. (1990) *Mol. Pharmacol.* **37**, 182–191.
26. Sabzevari, O., Hatcher, M., Kentish, P., O'Sullivan, M., and Gibson, G. G. (1996) *Toxicology* **106**, 19–26.
27. Mwinga, A., Hosp, M., Godfrey-Faussett, P., Quigley, M., Mwaba, P., Mugala, B. N., Nyirenda, O., Luo, N., Pobee, J., Elliott, A. M., McAdam, K. P., and Porter, J. D. (1998) *AIDS* **12**, 2447–2457.
28. Barry, M., Gibbon, S., Back, D., and Mulcahy, F. (1997) *Clin. Pharmacokinet.* **32**, 194–209.